Understanding Bacterial Virulence Genes and Mechanisms of Host Response to Insect-Mediated Citrus Huanglongbing

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ABSTRACT

A summary is provided for research progress in studying genomics and proteomics of molecular pathogen-host interactions for citrus huanglongbing (HLB), a destructive disease of citrus that presents a major threat to the citrus industries in US as well as other citrus production regions in the world. The disease is associated with a gram-negative, phloem-limited, insect-vectored, unculturable prokaryote: '*Candidatus* Liberibacter spp', that belong to the *Rhizobiaceae* family of α -Proteobacteria. Despite the fact that Koch's postulates have not been fulfilled, a considerable progress has been made in understanding molecular basis of HLB disease since the publication of HLB-associated Liberibacter genome. Annotation of the Liberibacter genome sequence has provided insights into the genetic basis of the virulence, physiological and metabolic capabilities of this organism. Functional determination of key virulence genes will permit researchers to design and develop a novel gene-based therapeutic treatment to control the disease. Since most commercial citrus cultivars are susceptible to HLB, understanding the molecular mechanisms of host response is crucial for the development and deployment of HLB-resistant citrus varieties. A long term and sustainable management of HLB is likely to be based on integrated strategies including removal or reduction of vectors or inocula, and the improvement of host resistance to HLB-associated Liberibacters and psyllid vectors.

Keywords: huanglongbing, *Candidatus* Liberibacter asiaticus, proteomics, virulence genes, host response

INTRODUCTION

Huanglongbing (HLB), also known as greening, is a destructive disease of citrus

worldwide that severely reduces productivity resulting in catastrophic economic losses [8, 20]. Generally, almost all commercial citrus cultivars are susceptible to HLB. There are some citrus cultivars or hybrids appear to be tolerance to HLB [43]. HLB-affected trees begin to decline within a few years of infection, produce reduced yields, poor quality fruit, and may die or become otherwise unproductive. HLB was first reported in Asian countries in the 1870s [30]. While Koch's postulates are yet to be determined, the etiology of the disease has been associated with '*Candidatus* Liberibacter spp', a group of Gram-negative, fastidious, phloem-limited α-proteobacteria. Taxonomically, there are three HLB-associated species, namely: '*Candidatus* Liberibacter asiaticus' (Las), '*Ca.* L. africanus' and '*Ca.* L. americanus', which are based on their presumptive origins from the Asian, African and American continents, respectively [8]. Among these three Liberibacter species, Las-associated HLB is the most prevalent and has been associated with increasing economic losses to citrus production worldwide. Since the first report of HLB in Florida in 2005, the disease has been observed in most citrus growing counties in Florida as well as in other citrus growing states in U.S. including recent occurrences in Texas and California [27, 28]. Las is transmitted and disseminated naturally by the Asian citrus psyllid (*Diaphorina citri*). The disease can also be transmitted by grafting with HLB-affected citrus plants or by dodder plants [8]. There is a latency period between times of infection and symptom development, which greatly complicates control strategies [20] making it crucial to develop fast, reliable and efficient methods for the early detection of infected plants.

Due to the fastidious nature of the Liberibacter, standard microbiological methods could not be directly applied for the causative agent study, thus, the details of etiology of the disease are limited. '*Candidatus* Liberibacter' associated diseases are usually present in very low titers and uneven distribution in plant hosts [17, 31], therefore attempts to obtain the complete Liberibacter genome directly using HLB-affected plants tissue failed. This difficulty was overcome by using a high through-put next generation deep sequencing technology in combination with the whole genome amplification approach [17]. The availability of Las genome has provided insight into the genetic basis of the virulence, physiological and metabolic capability of this organism. Annotation of the Las genome facilitates the identification and functional determination of the virulence genes responsible for HLB.

Since almost all citrus cultivars are susceptible or highly susceptible to HLB, research efforts have also been emphasized on understanding the molecular basis of host

responses to HLB. An important aspect of disease-associated plant-microbe interactions are the host responses during the disease development [36]. Identification of the host responses, especially at the infection or pre-symptomatic stage can be critical towards understanding the initial processes involved in disease development and could be exploited in the formulation of efficient disease management practices [12, 16]. At least three separate but complementary transcriptomics studies using microarray technology have been performed to elucidate the effects of Las infection on the total mRNA expression levels in tissues of sweet orange (*Citrus sinensis*) plants [2, 3, 25]. However, differential gene expression at the transcriptional (mRNA) level do not always correlate with differential gene expression at the translational (protein) level as posttranscriptional translational and/or posttranslational modifications; alternative splicing of mRNA transcripts and mRNA stability and interference factors play important roles in regulating gene expression [6, 22, 40, 46]. Proteins are the final products of gene expression and their expression levels directly correlate with cellular functions. In order to fully understand the molecular mechanisms involved in the response of citrus plants to Las-infection, it is therefore imperative to inquire beyond the transcriptional level and into the proteomic level of gene expression. To better understand Las associated HLB, we have developed research strategies focusing on: (1) genome-based analysis and functional determination of virulence genes of Las. (2) proteomic profile analysis of citrus in response to HLB.

Genome analysis and identification of putative virulence genes of Las

Motility is an important virulence factor in many pathogenic species. Several lines of evidence indicate that the activity of the flagellum may have an impact on virulence gene regulation [37]. Vascular disease pathogens require motility to establish a systemic infection and spread beyond the initially infected tissue. A complete set of genes involved in flagella biosynthesis were identified in the Las genome. In addition, genes involved in the biosynthesis and in the function of type IV pili are also present in Las genome. Sequence-based function analysis indicated that the functional domain of *pilG* is highly conserved in some members of proteobacteria included in *Xf* [45]. In *Xf*, *pilG* is a chemotaxis homologous gene in a Pil-Chp operon that regulates type IV pili. Interestingly, *Xf pilG* homologue gene was identified in the Las genome. Further gene structure analysis reveals that both share a functional REC superfamily domain. We hypothesize that the substitution of homologous Las *pilG* gene could restore a wild-type

phenotype of *Xf pilG* mutant. As Las is not yet culturable, conventional prokaryotic gene manipulation technique cannot be applied directly to this study. To circumvent this limitation, an *in vitro* heterologous gene expression system was employed.

Here we report a novel assay for characterization and functional determination of Las *pilG* gene. A site-directed deletion method was employed to generate a mutant *Xf pilG* [41]. To determine the function of Las *pilG* gene, a mutant *Xf pilG* was replaced with homologous gene of Las *pilG* (*XfΔpilG –R*-Las). In addition, the complementation of *Xf pilG* with native *Xf pilG* gene was also made (*XfΔpilG* –C). The function of twitching motility in *Xf pilG* and Las *pilG* were observed using microfluidic chambers. Microfluidic devices were fabricated using photo-lithographic procedures similar to that described previously [15, 33]. Specifically, *Xf* cells were collected from 4-6 day old grown on PD3 agar. Cell density was adjusted to an OD_{600nm} of 0.05 in PD3 broth. Cells were introduced through a separate inlet with 1 ml gas-tight syringes, and growth medium flow was maintained at 0.2 μL min^{-1} for 10 min to stabilize the system. Cell behavior was assessed microscopically using 40X phase-contrast optics from time-lapse image recordings using a SPOT-RT digital camera (Diagnostic Instruments, Inc., MI) controlled by MetaMorph Image software, NX version 2.0 (Universal Imaging Corp., PA). The number of cells exhibiting twitching motility was quantified using MetaMorph software. All experiments were conducted at room temperature.

Functional determination of Las *pilG*

In vitro growth curves showed that the biofilm formation by *XfΔpilG* was six-fold less than that of WT *Xf* after ten days of static incubation, measured by a crystal violet assay while genotype *XfΔpilG-C, ΔpilG–R*-Las had significantly a higher biofilm formation as compared with the mutant *Xf, XfΔpilG* (Fig.1), indicating that *XfΔpilG* has a reduced surface attachment ability, resulting in a reduced biofilm formation. On PD3 culture medium, colonies of the wild type, *XfΔpilG-C* and *ΔpilG–R*-Las exhibited a peripheral fringe, which is indicative of type IV pilus-mediated twitching motility by the bacteria, whereas *XfΔpilG* mutant exhibited a smooth and non-peripheral fringe phenotype (Fig. 2), implying that the *pilG* mutant phenotype results in.

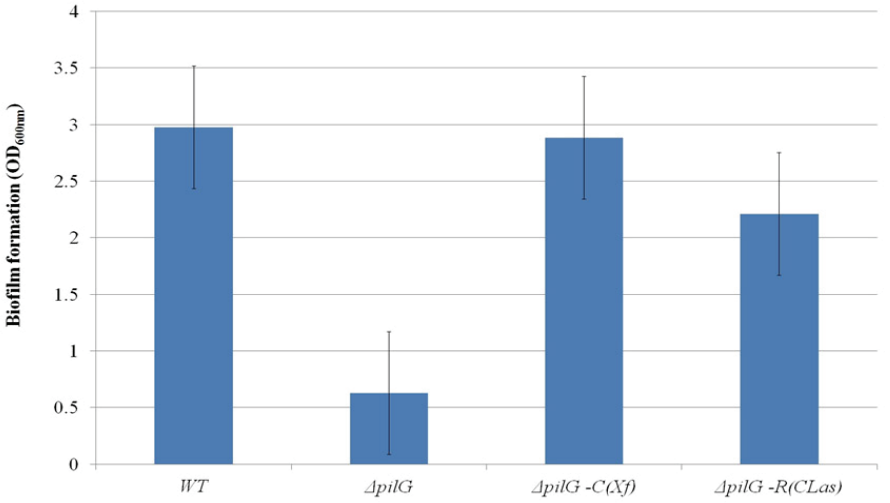

Figure 1. Quantitative assessment of biofilm formation of *X. fastidiosa* *XfΔpilG* (WT), mutant (*XfΔpilG*), complementation (*XfΔpilG-C)* and replacement (*XfΔpilG-R-Las)*. Means and standard deviations of three independent experiments.

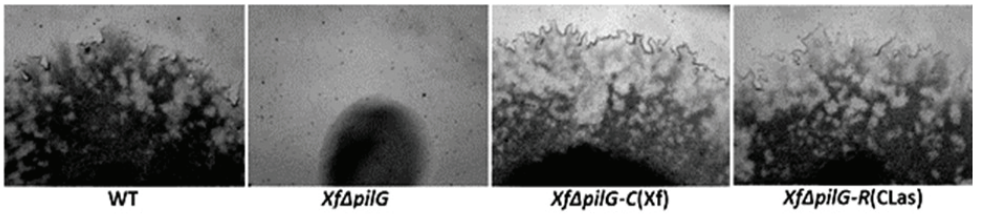

Figure 2. Colony characteristics of *X. fastidiosa* genotypes grown on PD3 agar. A peripheral fringe of colony was found in all genotypes except *XfΔpilG* mutant.

To confirm our results, *Xf* cells were assessed for motility in microfluidic flow chambers. Most *Xf* wild type cells exhibited twitching motility and developed cell aggregates in PD3 broth. In contrast, *Xf pilG* mutant cells had completely impaired twitching motility. However, twitching motility and the aggregation of cells were observed in both *XfΔpilG*-C and *XfΔpilG-R*-Las, suggesting *PilG* is required for cell twitching motility. Furthermore, this result indicates that pilus is highly conserved. *In vitro* heterologous gene expression experiments support the hypothesis that expression of type IV pili appears to be a requisite determinant of pathogenicity in Liberibacter associated disease. This study demonstrates the utility of heterologous gene expression

approach for functional characterization in unculturable Liberibacter.

Proteomic analysis of citrus host response to HLB

In this study, a comprehensive proteomic profile was constructed for proteomic analysis of citrus in response to HLB at asymptomatic and symptomatic stages. Two-year old grapefruit (*Citrus paradisi* cv. 'Duncan') plants were grown in an environment controlled, insect-proof greenhouse. Plants were either uninoculated or inoculated by side-grafting with 3-4 cm long bud sticks from PCR-confirmed HLB-affected (showing blotchy mottle and yellow shoots) lemon plants. Three months post-inoculation, 10-15 fully expanded leaves were collected from three individual plants each from the uninoculated or inoculated group. At this stage the infected plants were pre-symptomatic (no blotchy mottle, yellow shoots or symptoms of nutrient deficiency) but were PCR-positive for Las. Leaf samples from uninoculated or inoculated plants were grouped, respectively, as uninfected control for pre-symptomatic (UP) plants or infected pre-symptomatic (IP) plants. Six months post-inoculation, another set of 10-15 fully expanded leaves was collected from three individual plants each from the uninoculated or inoculated group. Successful inoculation was confirmed by PCR [(29)]. At this stage all of the inoculated plants were symptomatic for HLB and PCR-positive for Las. Leaf samples from uninoculated or inoculated plants were grouped, respectively, as uninfected control for symptomatic (US) plants or infected symptomatic (IS) plants.

Sample Preparation

The total leaf protein was extracted according to the early report [(35)]. Total protein extraction and quantification process were repeated three times generating three analytical replicates per plant. Total protein samples were then subjected two dimension electrophoresis separation and image analysis**.** Gel images were analyzed using the PDQuest software package (version 8.0, Bio-Rad, USA). A total of 36 gels were analyzed representing three analytical replicates per plant and three replicate plants per treatment. The gels were sorted into four groups namely: uninfected control for pre-symptomatic (UP) plants, infected pre-symptomatic (IP) plants, uninfected control for symptomatic (US) plants, or infected symptomatic (IS) plants. Gel spots were detected and matched so that a given spot had the same number across all gels. A master gel image containing matched spots across all gels was auto-generated.

Extensive analysis using the "Landmark" tool was used to resolve missed matches and spot volumes were normalized according to the total gel image density as suggested by the PDQuest software package. Only spots that had ≥10-fold increase over background and present in at least six of the nine gels per treatment as well as showed < 1.5 fold change ($P < 0.05$) compared to at least one other treatment group were considered to be differentially produced and further analyzed.

Protein mass spectrometry analysis

Prior to Protein mass spectrometry analysis, protein spots were manually excised (OneTouch Plus Spotpicker, The Gel company, USA) and digested with mass spectrometry grade trypsin in the presence of ProteaseMAX™ Surfactant according to the manufacturer's protocol (Promega, USA). For MALDI-TOF-MS/MS analysis (QSTAR XL Hybrid Quadrupole TOF LC/MS/MS System, Applied Biosystems, USA), the MASCOT search engine (Matrix Science, London, UK) was used to find matches of the PMF and MS/MS fragmentation spectra against a custom database containing entries for citrus (Citrus sinensis and Citrus clementina) available at http://www.citrusgenomedb.org/ and entries for grape (*Vitis vinifera*) available in the NCBI nonreduntant database. LC-MS/MS spectra were also searched via MASCOT against a custom citrus database. To gain functional information on identified proteins from MALDI-TOF and LC-MS/MS analysis, homology searches using $BLAST_P$ (http:www.ncbi.nlm.nih.gov/BLAST) was employed.

Effects of Las-infection on the leaf protein profile of pre-symptomatic and symptomatic grapefruit plants

There was no visible difference in leaf morphology between the uninfected control for pre-symptomatic stage plants and the infected pre-symptomatic stage plants but the uninfected control for symptomatic stage (US) plants was visibly different from the infected symptomatic stage (IS) plants. A total protein yield of over 10 mg g^{-1} was extracted from leaves and there was no significant difference in the total protein yield across treatments. A high resolution 2-DE separation of total leaf proteins from grapefruit plants was visualized in a p*I* range of 4-7 and M_r range of 10,000-150,000 (Fig. S1). Using PDQuest analysis software, over 700 spots per gel and over 440 reproducible spots within replicate gels were detected. Out of 191 differentially produced spots detected by PDQuest analysis, mass spectrometry analysis via

MALDI-TOF- or LC-MS was identified and summarized. An example of magnified view of the profiles of identified spots in representative gels from each treatment group is shown in Fig 3. Differentially expressed proteins were identified and categorized according to functional groups (Fig 4).

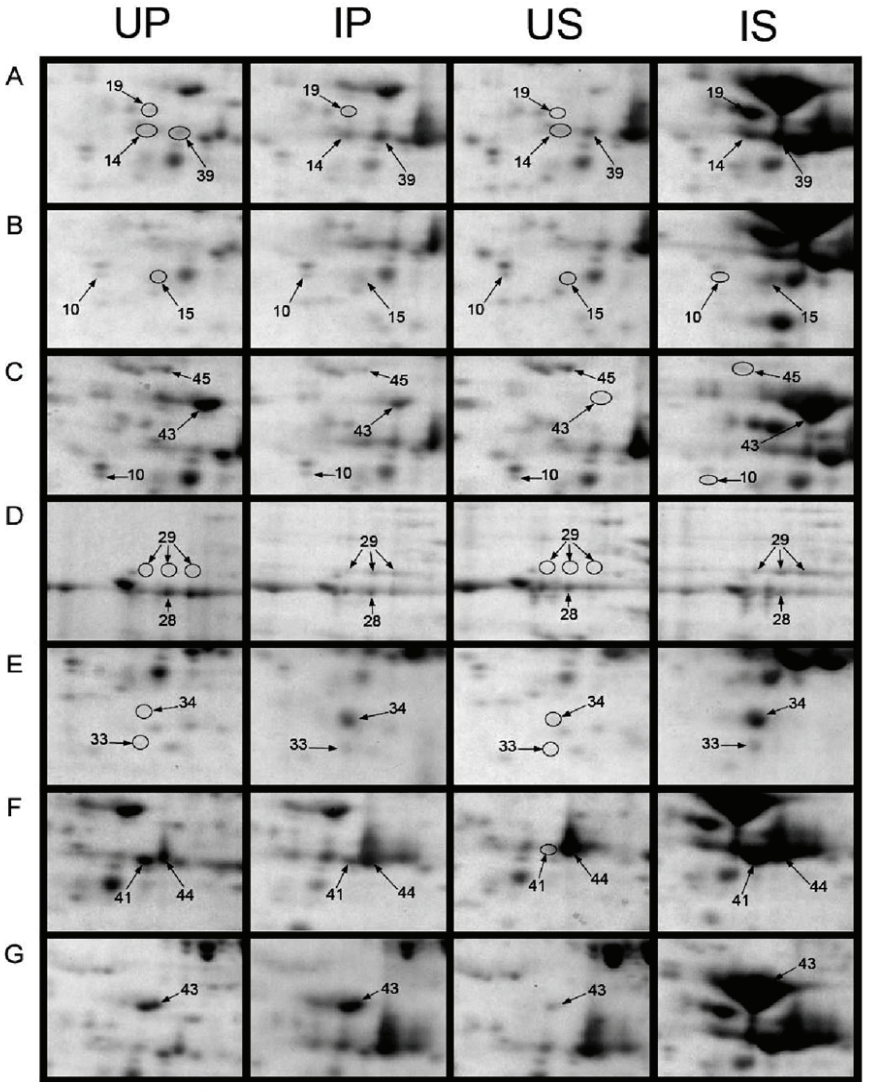

Figure 3. Differentially expressed protein spots were separated by 2D-electrophoresis and visualized by staining with Coomassie Brilliant Blue in UP (uninfected control for pre-symptomatic stage plants), IP (infected pre-symptomatic plants) US (uninfected control for symptomatic stage plants) and IS (infected symptomatic stage plants).

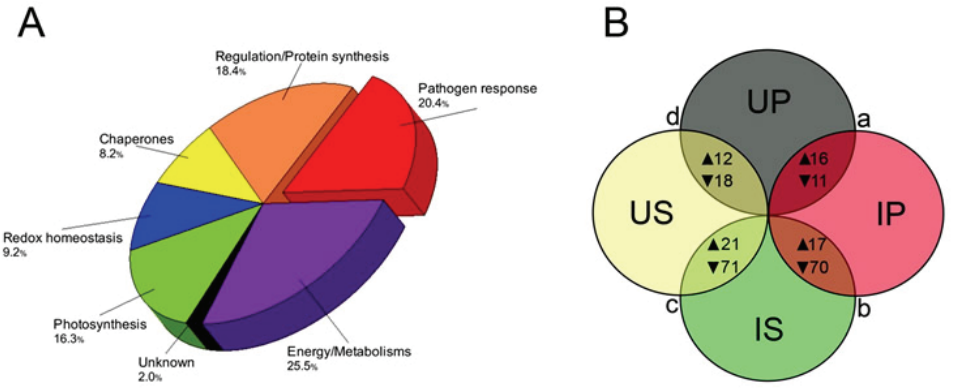

Figure 4. (**A**) Functional categories of identified differentially expressed protein spots from grapefruit plants that were Las-infected or uninfected and symptomatic or pre-symptomatic for HLB. (**B**) Venn diagram with intersections a, b, c and d, showing the number of identified protein spots that were significantly up- (▲) or down- (▼) regulated in comparison among four groups; IP, UP, IS and IP.

Pathogen response: Pathogenesis-related (PR) proteins are plant proteins that are induced in response to pathogen attack. However, several studies suggest that these proteins can also be induced by a variety of abiotic stresses, such as wounding and exposure to chemicals or heavy metals [9, 13, 35]. The PR-4 family of PR proteins consists of class I and class II chitinases, which differ by the presence (class I) or absence (class II) of a conserved N-terminal cysteine-rich domain corresponding to the mature hevein, a small antifungal protein isolated from rubber tree (*Hevea brasiliensis*) latex [10].

Lectin-like proteins are involved in vascular tissue differentiation [14] and are associated with the plugging of phloem sieve plates in response to wounding and defense against pathogens and insects [39]. Accumulation of Phloem protein 2 (PP2), a lectin-like protein, at the sieve plates together with phloem necrosis and blockage of the translocation stream was demonstrated by Kim [25] and Achor [1] in HLB-affected citrus plants. Furthermore, the deposition of PP2 with callose at the sieve plates played a role in the recovery of apple trees from apple proliferation disease caused by the phloem-limited pathogen '*Candidatus* Phytoplasma mali' [34]. Las, a phloem-limited bacterium, might induce the production of lectin-related proteins in host plants in order to inhibit phloem flow and accumulate photosynthates to nourish further bacterial growth as previously suggested. On the other hand host plants might induce the

production of lectin-like proteins as a defensive attempt to prevent the spread of Las by sealing off the sieve tubes. Additionally studies have demonstrated that lectin-like proteins are able to interact with RNA molecules, and are involved in the long-distance trafficking of macromolecules and may play a role in long-distance signaling in response to infection by plant pathogens [38].

In agreement with our results, a proteomics study by Fan et al. [18] also showed a Las-mediated up-regulation of miraculin-like proteins and gene transcripts in sweet orange plants. Recently, two distinct miraculin-like proteins, RlemMLP1 and RlemMLP2, were characterized in rough lemon (*Citrus jambhiri* Lush), and shown to have protease inhibitor activities as well as being involved in defense against pathogens [44]. During the development of citrus sudden death (CSD) disease, a miraculin-like protein was suppressed in susceptible plants but not in tolerant plants [11] . Increased levels of PR proteins as well as miraculin-like proteins was observed in leaves of *C. clementina* plants after infestation by the spider mite *Tetranychus urticae* or exposure to methyl jasmonate [32].

Redox homeostasis: Redox-homeostasis-related proteins are usually involved in the prevention of oxidative stress, which is induced by reactive oxygen species (ROS). ROS are by-products of electron transport and redox reactions from metabolic processes such as photosynthesis and respiration. The production of ROS is markedly increased under conditions of biotic or abiotic stress. We observed that Las-infection up-regulated the production of peroxiredoxins and Cu/Zn superoxide dismutase in IP and IS plants compared to their respective control plants. Additionally, we observed a Las-mediated up-regulation of a 2Fe-2S ferredoxin-like protein particularly in IP plants compared to UP plants. Antioxidants, such as superoxide dismutase (SOD), are among the most potent in nature in protecting living systems against oxidative stress. While the role of Cu/Zn SOD in HLB disease development in citrus plants has been previously demonstrated [4], this study provides novel evidence for the potential involvement of two other redox homeostasis-related proteins: peroxiredoxins and an uncharacterized 2Fe-2S ferredoxin-like protein. ROS are produced sequentially: superoxide (O_2^-) is the first reduction product of ground state oxygen and it can undergo spontaneous or SOD-catalyzed dismutation to H_2O_2, which is the second reactive product. Although, H_2O_2 is less reactive than superoxide, it is very diffusible and directly inactivates key cellular processes.

Regulation/Protein synthesis: Considering the general physiological decline that

accompanies HLB development, it is not surprising that proteins associated with regulation/protein synthesis including such as a 31 kDa ribonucleoprotein, EF-Tu, glutamine synthetase, an ATP-dependent zinc metalloprotease, a serine-type peptidase, nucleoside diphosphate kinase 1, and alanine aminotransferase 2, were markedly repressed by Las especially in IS plants compared to US plants. Interestingly, we observed a significant Las-mediated down-regulation of a transcription factor homolog (Btf3-like) protein and S-adenolsyl-L-methionine synthetase in IP and IS plants compared to control plants, suggesting that these proteins might be early targets of Las pathogenesis or an early susceptibility response by grapefruit during HLB development.

The RNA polymerase B transcriptional factor 3 (Btf3) was demonstrated to be associated with apoptosis in mammalian cells [7, 47] but its actual function in plants is not well understood. However, recently, Huh et al. [23] showed that silencing of Btf3 protein expression in *Capsicum annuum* and *Nicotiana benthamiana* plants led to reduced hypersensitive response (HR) cell death and decreased expression of some HR-associated genes. HR cell death upon pathogen infection has been described as a strategy devised by plants for inhibiting pathogen spread and obtaining systemic acquired resistance against further infection [24, 42]. Thus, an early Las-mediated reduction in the production of a Btf3-like protein in grapefruit plants would have facilitated the spread of the bacterium within the host.

Chaperones: Molecular chaperones [e.g. heat shock proteins (HSPs), chaperonins and peptidyl-prolyl cis-trans isomerases] are proteins involved in protein folding, refolding, assembly, re-assembly, degradation and translocation [2, 5, 19, 26]. It is, therefore, not surprising that the broad Las-mediated down-regulation of proteins associated with regulation/protein synthesis was accompanied by a corresponding down-regulation in the expression levels of chaperones including peptidyl-prolyl cis-trans isomerase, heat shock proteins and chaperonin-60 especially in IS plants compared to US plants.

CONCLUSION

HLB is currently one of the most destructive diseases of citrus and Las has been responsible for increasing economic losses in citrus production worldwide. Management of HLB remains elusive largely because the physiological and molecular processes involved in HLB-disease development are unresolved. In addition, almost all citrus cultivars tested so far are susceptible to HLB. Nevertheless, a great deal of research progress has been made in our understanding of the HLB since the publication of the

Las genome. Functional determination of key virulence genes will permit researchers to design and develop novel gene-based therapeutic treatment to control the disease. In addition, dissecting molecular mechanisms of host responses will advance our knowledge in understanding the genetic basis of HLB. A long term and sustainable management of HLB requires integrated strategies including the removal or reduction of vectors or inocula, and the improvement of host resistance to HLB-associated Liberibacters and psyllid vectors.

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